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Ultrastructural Histochemistry of *Marthasterias glacialis* (Echinodermata, Asteroidea) Gametes Before and After Fertilization

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Abstract. The gametes of the starfish *Marthasterias glacialis* were studied, both before and after fertilization, using several histochemical methods at the ultrastructural level. Results showed that the oocyte jelly coat (JC) could be morphologically differentiated into a fibrillar network of acidic polysaccharides, probably containing glycosaminoglycans (GAGs), and a loose component made of neutral polysaccharides that occupies the interstices of the former. Results also suggested that it is the fibrillar component of the JC to which the spermatozoon attaches and reacts. Staining of the acrosomal vesicle contents confirmed that the peripheral dense component attaches the spermatozoon to the JC, whereas the central and apical electron-lucent component coats the acrosomal process as it extends through the oocyte investments. The acrosomal process membrane was also shown to be negatively charged. Staining of cortical vesicles confirmed their tripartite compartmentalization: the spiral lamellae fused with the fertilization envelope (FE), transferring its staining to the inner layer of the FE, whereas the matrix could be subdivided into two components—a fibrillar mesh of acidic polysaccharides that fused with the FE and some amorphous aggregates that seem to become dispersed in the perivitelline space.

Introduction

The components of the oocyte jelly coat (JC) have not yet been differentiated at the morphological level, despite the importance of this information to the understanding of sperm-egg interaction. In the sea urchin, a sialoprotein and a fucose sulfate polysaccharide that induces the acrosomal reaction of the sperm have been isolated from the oocyte JC (SeGall and Lennarz, 1979), and morphological studies have confirmed the existence of two main JC polysaccharide components (Kidd, 1978; Jondeung and Czihak, 1982; Bonnell and Chandler, 1990). In the starfishes *Asterias amurensis* and *Asterina pectinifera*, a large sulfated glycoprotein, which induces the acrosomal reaction, and a high-mannose glycoprotein have been isolated from the oocyte JC (Hoshi *et al.*, 1990). At the light microscopical level, we have shown that the oocyte JC of the starfish *Marthasterias glacialis* is morphologically differentiated into two main polysaccharides (Sousa *et al.*, 1993), and here we confirm these findings at the ultrastructural level, showing their precise structure, distribution, and histochemical characteristics.

We have also shown that the acrosomal and cortical vesicles of *M. glacialis* gametes contain distinct morphological components that exhibit different destinies after being exocytosed (Sousa and Azevedo, 1986, 1987, 1988a, 1988b, 1989a, 1989b, 1990). Results of the present study further support the view that the compartmentalization of acrosomal and cortical vesicle contents serves to guide different components to different roles during fertilization.

Materials and Methods

Several specimens of *M. glacialis* were collected in the intertidal zone of the North Atlantic, 30 km north of Oporto, Portugal, and maintained in the laboratory in well-aerated seawater.

Small pieces of ovary and testis, free oocytes and fertilized eggs (Sousa and Azevedo, 1985) were processed for (1) ruthenium red (RR) staining according to Anderson

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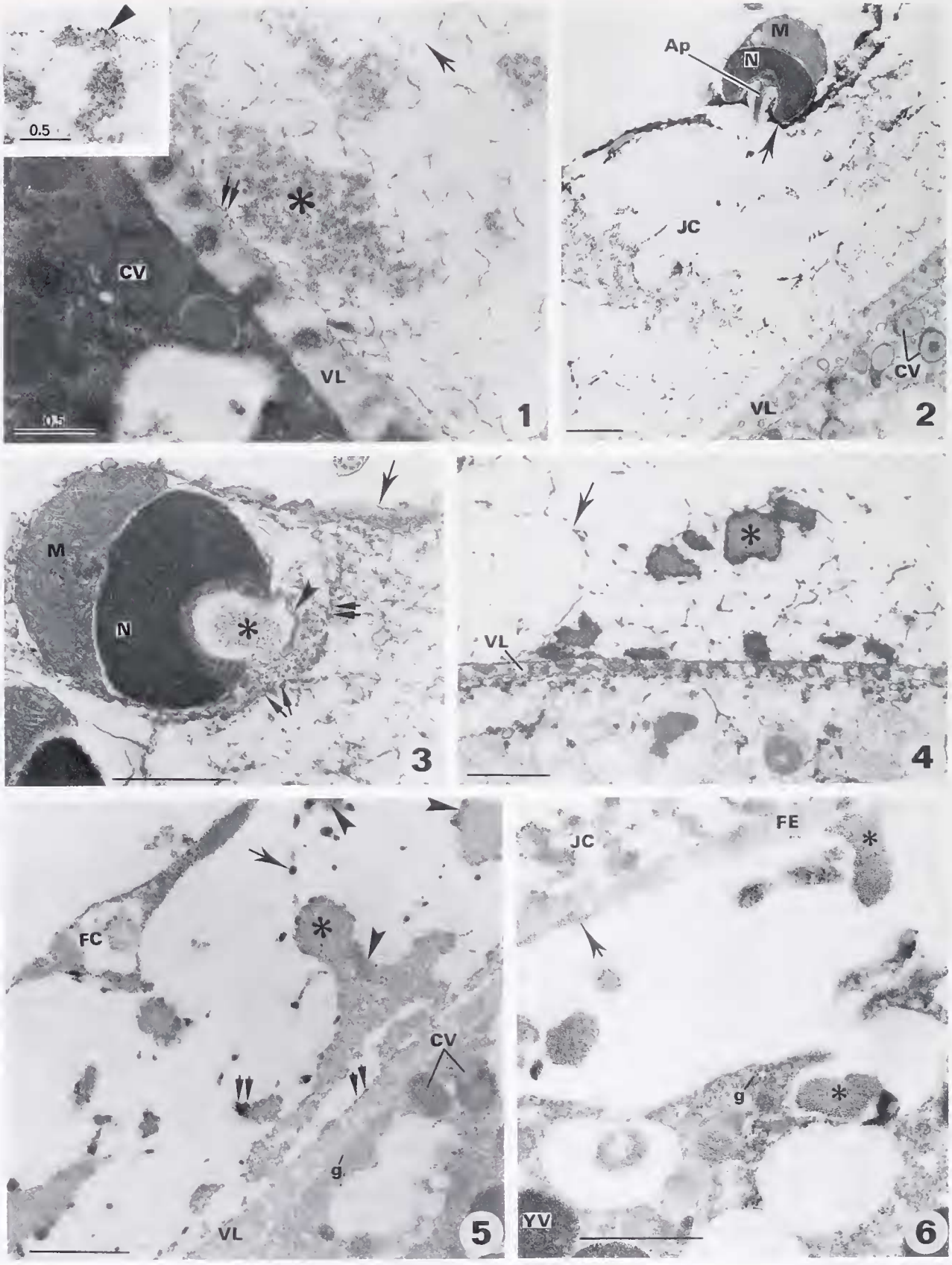


Figures 1–6. Transmission electron micrographs of *Marthasterias glacialis* oocytes with various stains. VL, vitelline layer; CV, cortical vesicles; JC, jelly coat; N, nucleus; M, mitochondrion; YV, yolk vesicles.

(1968); (2) alcian blue (Ab) at pHs 6.9 and 1, with the presence of $MgCl_2$, using the staining method of Crawford and Abed (1986); (3) high iron diamine-thiocarbohydrazide-silver proteinate (HID-TCH-SP) sequence according to Spicer *et al.* (1978) and Sannes *et al.* (1979); and (4) cationic cacodylate iron colloid (CCIC) as in Seno *et al.* (1983).

For histochemical studies on grid, the material was fixed in glutaraldehyde made in FSW-Na cacodylate (3:1), pH 7.2, washed, dehydrated, and embedded in Epon. Ultrathin sections were collected on gold grids and stained for (1) tannic acid-uranyl acetate (TA-UA) (Sannes *et al.*, 1978); (2) phosphotungstic acid (PTA)-chromic acid (PTA-CrA) (Rambourg, 1971; Rambourg *et al.*, 1969), PTA-HCl (Weinstock and Leblond, 1971), aqueous PTA (PTA-aq) (Krimer and Esponda, 1980) and PTA-acetone; (3) periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) (Thiéry, 1967); (4) periodic acid-silver methenamine (PA-SM) and PA-CrA-SM (Rambourg, 1967; Dawson and Filipe, 1976); (5) CCIC; and (6) HID.

Ultrathin sections were observed in a JEOL 100 CX II transmission electron microscope (TEM) operated at 60 kV.

For scanning electron microscopy (SEM), fixed oocytes were washed in 0.2 *M* Na-cacodylate pH 7.2, postfixed with 1% osmium tetroxide in the buffer for 2 h at 4°C, dehydrated in ethanol series, critically point dried in carbon dioxide, mounted on specimen stubs with double-coat tape, coated with gold, and examined in a JEOL 35 C SEM.

Results

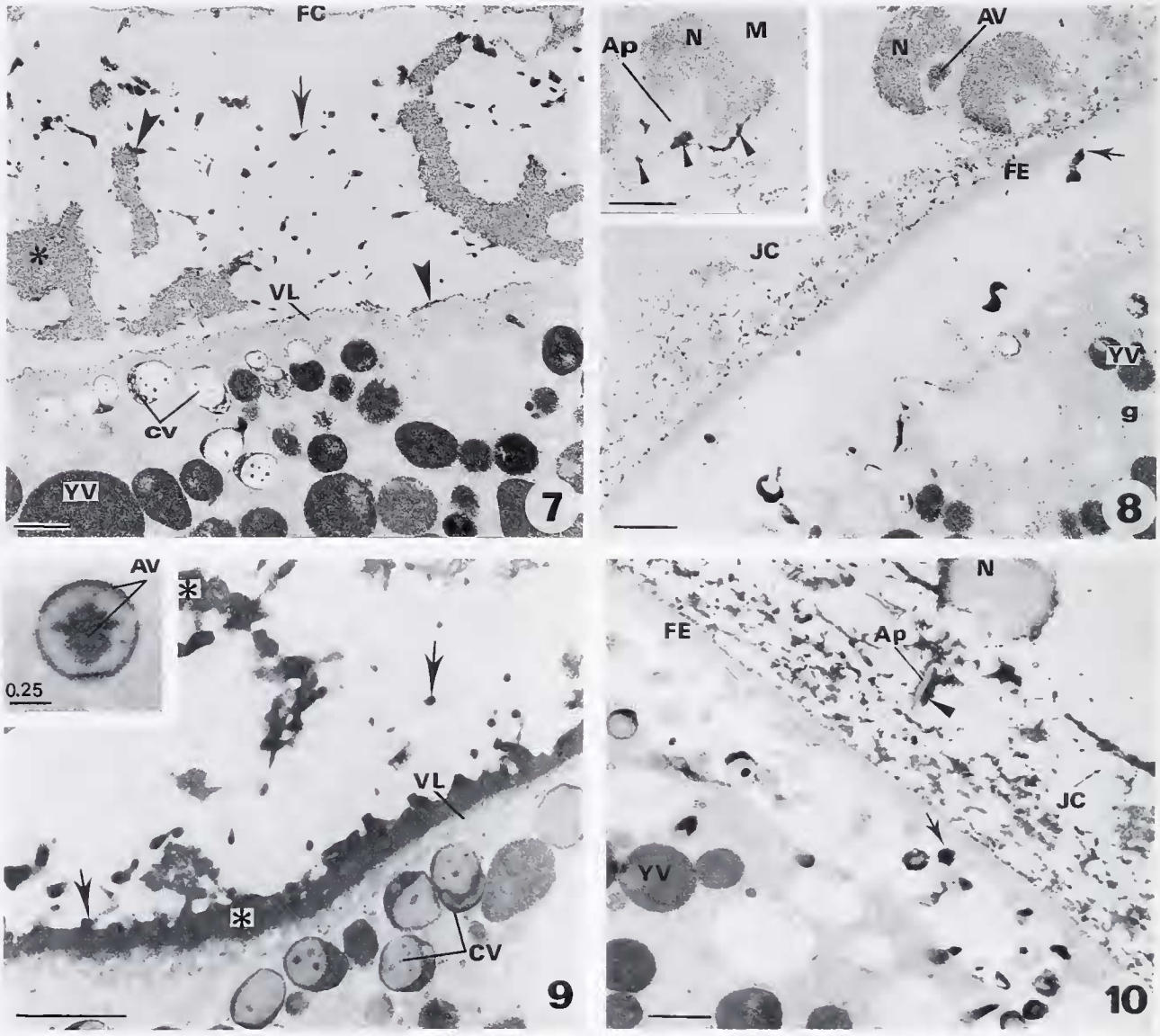
In the oocyte JC, RR stained a fibrillar material that delimits both the outer and inner aspects of the JC and spreads between some amorphous aggregates (Fig. 1). With insemination, the amorphous component dissolved, and the exocytosed peripheral dense component of the acrosomal vesicle, now stained by RR, attached the spermatozoon to the JC outer border (Fig. 2). Similar results were obtained with Ab pH 7 (Fig. 3). At pH 1, Ab stained the fibrillar component and the periphery of the amorphous aggregates of the JC (Fig. 4). The Thiéry technique stained some globular regions along the fibrillar component of the JC as well as its sites of attachment to the amorphous aggregates and vitelline layer, some regions of the periphery of the amorphous aggregates, and cortical vesicles (Fig. 5). After the cortical reaction, cortical vesicles transferred their staining to the undercoating of the FE (Fig. 6). Silver methenamine labeled the globular and adjacent parts of the fibrillar component of the JC as well as its sites of attachment to the amorphous aggregates and vitelline layer, some portions of the matrix of cortical vesicles (Fig. 7), and the sperm acrosomal vesicle (Fig. 8). With fertilization, no staining was transferred from cortical vesicles to the FE, whereas the stained acrosomal material attached the spermatozoon to the JC and coated the acrosomal process (Fig. 8). TA stained both components of the JC, some regions of the matrix of cortical vesicles, and the peripheral and central electron-lucent component of the acrosomal vesicle (Fig. 9). With fertilization, the stained acrosomal component coated the acrosomal process, and the stained material of cortical vesicles fused with the FE, although without transferring the staining to it (Fig. 10). Similar results were obtained with PTA, although the amorphous component of JC was more difficult to stain, and the acrosomal vesicle did not stain with PTA-HCl and PTA-Aq. No staining was found with the HID and HID-TCH-SP techniques. With the CCIC method, staining was observed only at pH 7. Unreacted oocytes did not show any staining (Fig. 11), but after the cortical reaction some regions of the matrix of cortical vesicles appeared stained, although the labeling was not transferred to the FE (Fig. 12). When applied on grid, CCIC stained the fibrillar component of the JC of unreacted oocytes (Fig. 13).

In the spermatozoon, CCIC staining disrupted the plasma membrane, which enabled the label to be found in the periphery of microtubules, in the pericentriolar

Figures 1–2. Ruthenium red stain (RR). In the unreacted oocyte (Fig. 1), RR stained a fibrillar material that delimits the outer (arrowhead) and inner (double arrow) regions of the JC and spreads between (arrow) some amorphous aggregates (*); bar = 0.5 μm. At fertilization (Fig. 2), the stained peripheral dense component (arrow) of the acrosomal vesicle attached the spermatozoon to the JC. Ap, acrosomal process; bar = 1 μm.

Figures 3–4. Alcian blue (Ab) stain; bar = 1 μm. At pH 7 (Fig. 3), Ab shows that the spermatozoon reacted over the Ab-stained inner fibrils (double arrows) and not at the large outer stained region (arrow) of the JC fibrillar component. Fused sperm acrosomal and plasma membranes (arrowhead); (*), precipitated acrosomal contents. At pH 1 (Fig. 4), Ab stained the fibrillar material (arrow) and the periphery of the amorphous (*) JC components.

Figures 5–6. The Thiéry staining technique; bar = 1 μm. In the unreacted oocyte (Fig. 5), stain was taken up by some globular regions along the JC fibrillar component (arrow) as well as its sites of attachment (double arrows) to the amorphous aggregates (*) and VL, some regions at the periphery of the amorphous aggregates (arrowheads), and the CV. FC, follicular cell; g, glycogen. After the cortical reaction (Fig. 6), CV contents (*) transferred their stain to the undercoat (arrow) of the fertilization envelope (FE).



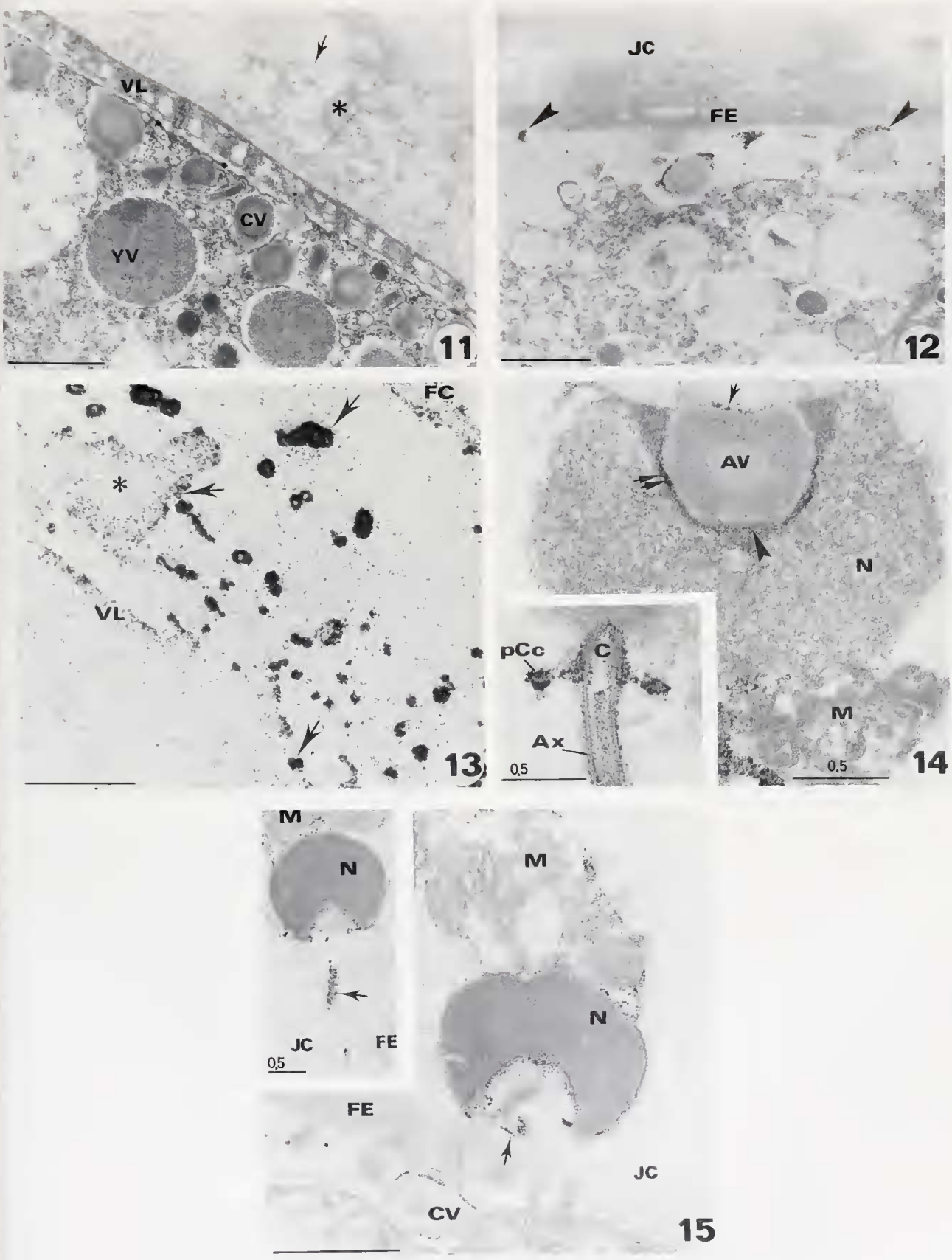
Figures 7–10. Transmission electron micrographs of *Marthasterias glacialis* oocytes with various stains. VL, vitelline layer; CV, cortical vesicles; JC, jelly coat; YV, yolk vesicles; N, nucleus; M, mitochondrion, Ap, acrosomal process; FE, fertilization envelope.

Figures 7–8. Periodic acid-silver methenamine (PA-SM) stain; bar = 1 μm. In the unreacted oocyte (Fig. 7), stain was taken up by the globular and adjacent parts of the JC fibrillar component (arrow), as well as by its sites of attachment (arrowheads) to the amorphous aggregates (*) and VL, and by some portions of CV. With fertilization (Fig. 8), no stain was transferred from the CV (arrow) to the FE, whereas the stained acrosomal contents (AV) attached the sperm to the JC and coated the Ap (arrowheads); g, glycogen.

Figures 9–10. Tannic acid-uranyl acetate (TA-UA) stain; bar = 1 μm. In the unreacted oocyte (Fig. 9), TA-UA stained the fibrillar (arrows) and amorphous (*) JC components, some portions of the CV, and the acrosomal vesicle (AV) peripheral and central electron-lucent components. Inset = 0.25 μm. With fertilization (Fig. 10), the AV components (arrowhead) coated the Ap, whereas the component of the CV (arrow) fused with the FE without transferring any staining to it.

complex, and in the acrosome (Fig. 14). The label on intact plasma membrane was minimal, but after the acrosomal reaction the membrane of the acrosomal process appeared intensely labeled (Fig. 15).

When observed by SEM, the JC appeared as a complex network of fibrillar materials with different thicknesses, which probably correspond to the fibrillar component observed by TEM (Fig. 16).



Figures 11–15. Transmission electron micrographs of *Marthasterias glacialis* oocytes and spermatozoa stained with cationic cacodylate iron colloid (CCIC) pH 7. VL, vitelline layer; CV, cortical vesicles; JC, jelly coat; YV, yolk vesicles; N, nucleus; M, mitochondrion; FE, fertilization envelope; bar = 1 μm; insets = 0.5 μm.

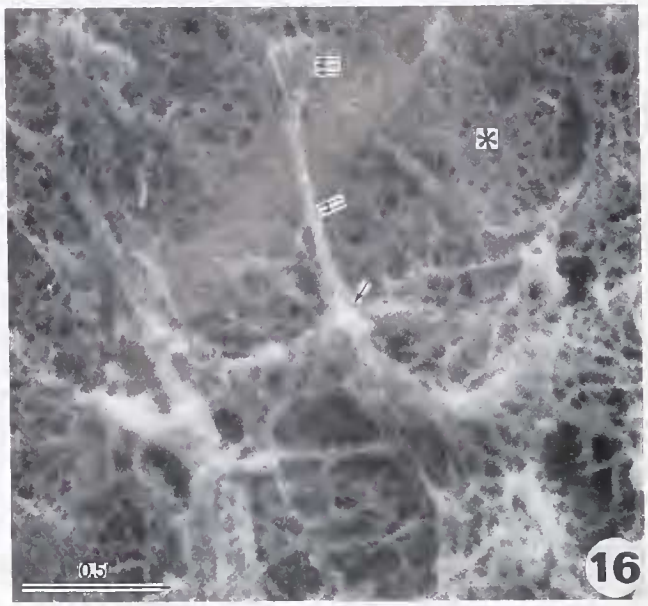


Figure 16. When observed by scanning electron microscopy, the jelly coat was seen to be formed by large dense fibrous structures that progressively branched (arrow to triple arrow) to give origin to the inner fine fibrillar network (*). Bar = 0.5 μm.

Discussion

Despite the intensive biochemical characterization of the echinoderm JC, the differentiation of its components at the ultrastructural level is not well known. In the sea urchin, RR staining distinguished between amorphous and fibrillar components (Kidd, 1978), and Bonnell and Chandler (1990) demonstrated, by platinum replicas, that the fibrillar material corresponded to the fucose-sulfate-rich polysaccharide responsible for inducing the acrosomal reaction of the spermatozoon (SeGall and Lennarz, 1979).

In the present study, RR and Ab stained a fibrillar material in the JC of *M. glacialis* oocytes; this material forms a branched matrix all around the oocyte and contains amorphous aggregates between its interstices. At fertilization, the spermatozoon bound and reacted over the fibrillar material, whereas the amorphous aggregates dissolved as the acrosomal process penetrated the jelly layer. RR is known to precipitate GAGs, acidic polysaccharides, and polypeptides, but not neutral polysaccharides (Luft, 1966), whereas at neutral pH and in the presence of $MgCl_2$, Ab selectively stains GAGs (Reale *et al.*, 1986). Thus the amorphous aggregates probably correspond to neutral polysaccharides and the fibrillar component to acidic polysaccharides, with the possibility that this latter material may contain GAGs. The acidic nature of the fibrillar material was also demonstrated by the presence of surface negative charges, as shown by CCIC staining and by the fact that it bound calcium (Sousa and Azevedo, 1989b). TA and PTA techniques for complex carbohydrates stained both JC components. The absence of staining with HID argues against the presence of sulfated groups in the JC, but this result can also be due to the masking or inaccessibility of those groups. Staining with the Thiéry and silver methenamine techniques for acid glycoproteins showed a more complex composition of both JC components, because only some regions appeared stained. Although fixation and dehydration is likely to lead to artifacts such as shrinkage and solubilization of material and the jelly structure could have been altered during these processes, the effects might have been lessened by RR and Ab stainings. Therefore, the fibrillar and amorphous components of the JC can be assumed to correspond to the major structural components of the jelly layer. In conclusion, results showed that the JC of the starfish oocyte can be morphologically differentiated into two main polysaccharides, thus confirming the previous light microscopical and biochemical (Hoshi *et al.*, 1990; Sousa *et al.*, 1993) findings. The putative existence of GAGs in the JC of *M. glacialis* oocytes is further confirmed in Sousa *et al.* (1993).

In *M. glacialis*, the acrosomal vesicle of the spermatozoon contains ATPase and calcium in the peripheral dense component, and acid and alkaline phosphatases in the central and apical electron-lucent component (Sousa and Azevedo, 1986, 1988a, 1989b). In the present work, this differentiation was further confirmed because RR, Ab, and PA-SM stained the peripheral dense component, which bound the sperm to the JC, whereas TA, PTA, and PA-SM stained the central and peripheral electron-lucent component, which coated the acrosomal process. Simi-

Figure 11. The unreacted oocyte did not show any staining in the fibrillar (arrow) and amorphous (*) JC components.

Figure 12. After cortical reaction, some regions of the CV appeared stained (arrowheads), although the labeling was not transferred to the FE.

Figure 13. On-grid staining of oocytes labeled the fibrillar (arrows) JC component. FC, follicular cell.

Figure 14. In the unreacted sperm, leaked plasma membranes allowed staining of the periphery of microtubules of centrioles (C) and axoneme (Ax), the pericentriolar complex (pCc), the acrosomal-plasma membrane contact zone (arrow), the basolateral periacrosomal material (double arrow), and the inner acrosomal vesicle (AV) membrane (arrowhead).

Figure 15. After the acrosomal reaction, the acrosomal process membrane appeared labeled (arrow).

larly, the oocyte cortical vesicles have also been compartmentalized into spiral lamellae, which contain peroxidase activity, and a matrix that was further subdivided into a mesh-like structure that stains with RR, Ab, and $AgNO_3$ as well as for acid and alkaline phosphatase activities, and contains amorphous aggregates within its holes (Sousa and Azevedo, 1987, 1988b, 1989a, 1990). Although both the mesh-like component and the spiral lamellae fuse with the FE, only the staining carried by the latter is transferred to the inner layer of the FE. Our present results confirmed this tripartite structural and functional nature of cortical vesicles, with acidic polysaccharides and negative charges found to be concentrated in the mesh-like component.

Acknowledgments

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